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Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: Significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase

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ABSTRACT **The gene encoding cytosine deaminase (CD) has been expressed in the human colorectal carcinoma cell line WiDr. Metabolism studies confirm that tumor cells expressing CD convert the very nontoxic prodrug 5-fluorocytosine (5FCyt) to 5-fluorouracil (5FUra) and 5FUra metabolites. Tumor xenografts composed of CD-expressing cells can selectively generate tumor levels of >400 μM 5FUra when the host mouse is dosed with nontoxic levels of 5FCyt. The selective metabolic conversion of 5FCyt to 5FUra in CD-expressing tumor cells results in the inhibition of thymidylate synthase and incorporation of 5FUra into RNA. 5FUra is also liberated into the surrounding environment when CD-expressing tumor cells are treated with 5FCyt. The liberated 5FUra is able to kill neighboring, non-CD-expressing tumor cells *in vitro* and *in vivo*. Most importantly, when only 2% of the tumor mass contains CD-expressing cells (98% non-CD-expressing cells), significant regressions in all tumors are observed when the host mouse is dosed with nontoxic levels of 5FCyt.**

We have previously reported our efforts to develop a therapeutic approach using gene transfer technology for the treatment of primary and metastatic tumors (1–4). This approach, virus-directed enzyme/prodrug therapy, exploits transcriptional differences between normal and neoplastic cells to selectively kill cancer cells. An artificial chimeric gene is created that is composed of tissue-specific transcriptional regulatory sequences linked to the coding domain of a nonmammalian enzyme. The nonmammalian enzyme can metabolically activate a nontoxic prodrug to a cytotoxic anabolite. If the tissue-specific regulatory sequences are from a tumor-associated marker gene such as the carcinoembryonic antigen gene (5, 6), the prostate-specific antigen gene (7, 8), or the α-fetoprotein gene (9, 10), then the artificial chimeric gene will result in tumor-specific expression of the nonmammalian enzyme and, consequently, in tumor-specific production of the cytotoxic metabolite.

A critical issue influencing the therapeutic benefit of this approach will be the efficiency of gene transfer into a solid tumor. However, the efficacy of this gene therapy approach is dependent not only on the efficiency of gene transfer but also on the enzyme/prodrug system. Various enzyme/prodrug systems have been reported (1–3, 11–14). A prodrug that is metabolically converted into a toxic anabolite that is not readily diffusible from one tumor cell into another will require very high gene transfer efficiency. However, if the toxic anabolite is readily diffusible, then the efficiency of gene transfer into the tumor mass may be quite low but still be able to achieve a significant therapeutic effect.

To develop this approach for colorectal carcinoma, we have cloned the bacterial enzyme cytosine deaminase (CD) [refs. 2 and 15; see also an independent report of Danielsen *et al.* (16)] and have demonstrated significant *in vivo* antitumor effects on human colorectal tumor xenografts transduced with CD when the host animal is treated with the prodrug 5-fluorocytosine (5FCyt) (2, 3). CD was chosen because 5FCyt is very nontoxic in man (17, 18); the toxic anabolite 5-fluorouracil (5FUra) is the drug of choice for colorectal carcinoma (19–21); and 5FUra can readily diffuse into and out of cells by nonfacilitated diffusion (22). We have hypothesized that to produce a significant therapeutic benefit, the required efficiency of CD gene transfer into a tumor mass may be quite small, since tumor cells which selectively express CD may produce significant amounts of 5FUra which can readily diffuse into and kill neighboring untransduced tumor cells.

To test this hypothesis, we have now determined the metabolic profile *in vitro* and *in vivo* of 5FCyt in human tumor cells transduced with the CD gene. Most importantly, we show that solid tumors require only a very small percentage of tumor cells expressing CD to generate very significant antitumor effects when the host animal is treated with nontoxic levels of 5FCyt.

MATERIALS AND METHODS

Materials. [6-^{3}H]5FCyt (11 Ci/mmol; 1 Ci = 37 GBq) and 2′-deoxy[5-^{3}H]uridine (22 Ci/mmol) were obtained from Moravek Biochemicals (La Brea, CA). The human colorectal carcinoma cell line WiDr was transduced with a CD expression vector, and cell growth rate and IC_{50} were determined as described (1–3).

Tumor Studies with Mice. Tumor cells (10^7) were implanted s.c. in 0.5 ml of saline. Tumor weights were estimated as described (3). Procedures were performed with approved protocols and in accordance with recommendations for proper care and use of laboratory animals (23).

Analytical Methods. Cells were plated at 10^5 per 4 ml per 35-mm well. After incubation with [6-^{3}H]5FCyt (30 μM; final specific activity, 166 mCi/mmol), cell-conditioned medium was removed and 2 ml of 60% (wt/vol) methanol was added. Cells were lysed by freeze–thawing and centrifuged for 10 min at 8000 × *g*. The conditioned medium and the cell extract supernatant were evaporated to dryness, dissolved in deionized water (1 ml for medium; 100 μl for extract). Aliquots (20 μl) of each sample were analyzed by HPLC on a Partisphere C_{18} column (4.6 mm × 250 mm; Whatman). The samples were

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Abbreviations: CD, cytosine deaminase; 5FCyt, 5-fluorocytosine; 5FUra, 5-fluorouracil; TS, thymidylate synthase.

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eluted isocratically with 50 mM ammonium phosphate/0.1% triethylamine, pH 2.1 (buffer A), for 10 min, followed by a 10-min linear gradient of 0–60% acetonitrile in buffer A. The UV absorbance and radioactive content of the column effluent were monitored with a model 486 tunable absorbance monitor (Waters) and a Flo-One/B radioactive flow detector (Radiomatic Instrument and Chemical Co., Meriden, CT).

For determination of 5FCyt and 5FUra in tumors, mice received 500 mg of [6-^{3}H]5FCyt/kg of body weight (100 μCi per animal), tumors were removed, and extracts were made with 10 volumes of cold 60% (wt/vol) methanol. Extracts were evaporated to dryness, dissolved in deionized water to 1 or 2 ml/g of original tumor weight, and analyzed by HPLC as described above.

To measure radioactivity incorporated into RNA and DNA, nude mice bearing WiDr or WiDr/CD tumor xenografts were dosed i.p. with 500 mg of [6-^{3}H]5FCyt/kg of body weight (50 μCi per animal). After 24 hr, tumor extracts were prepared and radioactivity in RNA and DNA fractions was determined (24).

For thymidylate synthase (TS) activity, a modification of the method of Yalowich and Kalman was used (25). Cells at 2×10^5 in 2 ml per 35-mm well were incubated for 3 hr with either 5FCyt or 5FUra before 50 μM [5-^{3}H]2'dUrd (final specific activity, 4.4×10^6 dpm/nmol; final concentration, 1 μM) was added. At various times 0.2 ml of medium was removed and 60 μl of 20% (wt/vol) trichloroacetic acid was added followed by 0.2 ml of 10% activated charcoal. This mixture was vortexed and centrifuged at 10,000 × *g* for 5 min, and 0.25 ml of the supernatant was assayed by liquid scintillation counting.

RESULTS AND DISCUSSION

Metabolism of 5FCyt to 5FUra *in Vitro*. We have previously demonstrated that expression of CD in WiDr cells results in an ≈1000-fold shift in the IC_{50} for 5FCyt, from 26,000 μM in parental WiDr cells (WiDr) to 27 μM in WiDr cells expressing CD (WiDr/CD) (3). To establish that the increased sensitivity of WiDr/CD cells to 5FCyt was due to the conversion of 5FCyt to 5FUra, WiDr and WiDr/CD cells were cultured in the presence of 30 μM [6-^{3}H]5FCyt. Cells and conditioned media were harvested 24, 48, and 96 hr following addition of 5FCyt. HPLC analysis of WiDr cell extracts detected only a single peak identified as 5FCyt at all time points (Fig. 1*A*). In contrast, analysis of WiDr/CD cell extracts at the 24-hr time point detected peaks that were coeluted with 5FCyt, 5FUra, 5FUrd, and 5FdUrd (Fig. 1*A*). By 48 hr, an additional peak

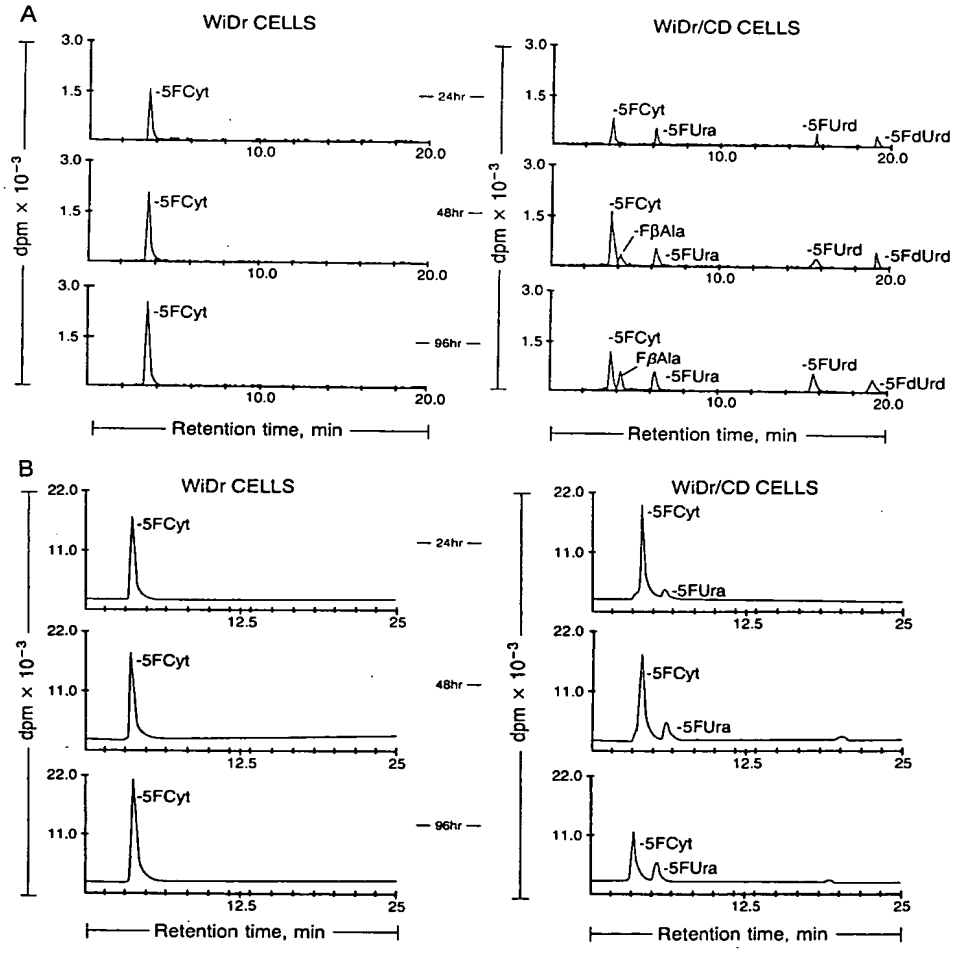

FIG. 1. *In vitro* metabolism of 5FCyt. HPLC tracing of radiolabeled metabolites detected in cell extracts (Fig. 1*A*) or cell-conditioned media (Fig. 1*B*) of WiDr and WiDr/CD cells cultured for 24, 48, or 96 hr in the presence of [6-^{3}H]5FCyt. FβAla, fluoro-β-alanine.

was detected that was assumed to be FβAla, since it was eliminated by pretreatment with a uracil reductase inhibitor.

Conditioned media were also analyzed for the presence of 5FCyt, 5FUra, and 5FUra metabolites (Fig. 1*B*). Only 5FCyt was detected in medium from WiDr cells. Medium from WiDr/CD cells showed 5FCyt, 5FUra, and possibly other minor metabolites. Thus, 5FCyt is metabolically very stable in parental WiDr cells; 5FCyt is converted to 5FUra selectively in WiDr/CD cells, and 5FUra is liberated from WiDr/CD cells in very significant quantities.

Metabolism of 5FCyt to 5FUra *in Vivo*. Nude mice bearing either WiDr or WiDr/CD tumor xenografts were dosed with 500 mg of [6-^{3}H]5FCyt/kg (100 μCi per animal). At 30 min, there was no detectable 5FUra in WiDr tumor extracts (Fig. 2). At 60 min, there was ≈18 μM 5FUra in WiDr tumor extracts, which represents only 1% of the total radioactivity in those samples. Based on the lack of conversion of 5FCyt to 5FUra in WiDr cells *in vitro* (see Fig. 1*A*), the 5FUra content in the WiDr tumor extract probably resulted from plasma 5FUra arising from the conversion of 5FCyt to 5FUra by the gut flora (26). In contrast, WiDr/CD tumors at 30 and 60 min had significantly less 5FCyt (77% and 68% of the total radioactivity in those samples, respectively) and significantly higher 5FUra (18% and 27% of the total radioactivity in those samples, respectively) (Fig. 2). The levels of 5FUra represent ≈260 μM and 435 μM at 30 min and 60 min, respectively. Thus, 5FCyt is converted to 5FUra *in vivo* by tumors containing CD-expressing cells.

TS Inhibition and Adduct Formation in Tumor DNA and RNA. Cell death following treatment with 5FUra is thought to result from 5FUra anabolism to (*i*) 5FdUMP, which can inhibit TS and thus block DNA synthesis; and (*ii*) 5FUTP incorporation into RNA, thus disrupting the function of RNA. Incorporation of 5FUra anabolites into DNA can occur before complete inactivation of TS, but these effects are hypothesized to be a minor component of the antitumor and cytotoxic effects of 5FUra (for reviews see refs. 27 and 28).

WiDr and WiDr/CD cells were preincubated for 3 hr in the presence of 5FCyt or 5FUra at concentrations approximating the IC_{50} and 3 times the IC_{50}. TS enzymatic activity was then measured over time by the addition of [5-^{3}H]dUrd. WiDr and WiDr/CD cells had statistically identical levels of TS activity. As expected, 5FUra treatment inhibited TS activity in both WiDr and WiDr/CD cell lines (Fig. 3 *A* and *B*). The inhibition was dose dependent and proportional to the 5FUra IC_{50}. 5FCyt had no significantly inhibitory effect on TS activity in WiDr cells at the highest concentration tested (100 μM) (Fig. 3*C*) but did significantly inhibit TS activity in WiDr/CD cells (Fig. 3*D*). The inhibition was, likewise, dose

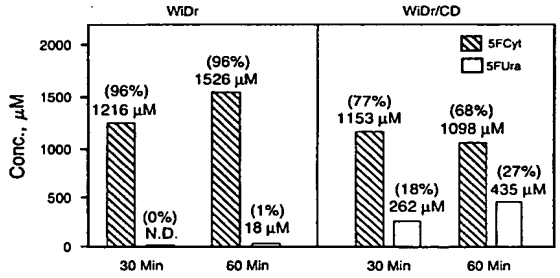

FIG. 2. 5FCyt and 5FUra detected in tumor extracts in mice treated with 5FCyt. Nude mice bearing WiDr (*Left*) or WiDr/CD (*Right*) cell-derived tumor xenografts were treated with [6-^{3}H]5FCyt. At 30 or 60 min, tumors were removed and 5FCyt (hatched bars) and 5FUra (open bars) were determined by HPLC. Numbers in parentheses represent percent of the total radioactivity in that sample. N.D., not detectable.

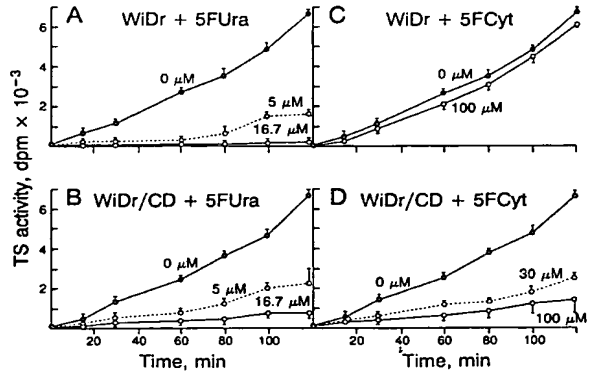

FIG. 3. TS activity in WiDr and WiDr/CD cells treated with 5FUra or 5FCyt. TS activity was determined in WiDr cells treated with either 5FUra (*A*) or 5FCyt (*C*) or in WiDr/CD cells treated with either 5FUra (*B*) or 5FCyt (*D*). Each point is an average of three independent determinations. SE bars are included where graphically possible. For *A*, *B*, and *D*, treated samples (○) were significantly different from untreated samples (○) ($P \leq 0.05$). For *C*, treated samples (○) and untreated samples (○) were not significantly different ($P \geq 0.6$).

dependent and proportional to the 5FCyt IC_{50} in WiDr/CD cells.

Mice bearing either WiDr-derived or WiDr/CD-derived tumor xenografts were treated with 500 mg of [6-^{3}H]5FCyt/kg (50 μCi per mouse). Twenty-four hours later, incorporation of radioactivity into tumor DNA and RNA was determined (Table 1). Low incorporation into both DNA and RNA was detected in WiDr-derived tumors. This finding was not unexpected, since 5FCyt was administered at 500 mg/kg and there may be some conversion of 5FCyt to 5FUra mediated by the gut flora (26). However, in WiDr/CD-derived tumors, incorporation of radioactivity into RNA was ≈50-fold greater than that observed in WiDr-derived tumors. As expected, there was very little incorporation of radioactivity into DNA. The very low but measurable incorporation of radioactivity into DNA in WiDr/CD-derived tumors was presumably due to anabolism of 5FdUMP to 5FdUTP and incorporation of 5FdUTP into DNA. Taken collectively, these data illustrate that 5FCyt is converted to 5FUra in WiDr/CD cells. Generation of 5FUra in these cells results in the inhibition of TS as well as adduct formation in RNA.

Neighbor Cell Killing *in Vitro*. The presence of 5FUra in the conditioned medium from WiDr/CD cells that were incubated with 5FCyt suggests that a neighbor cell killing effect would occur in cells near those expressing CD. This hypothesis was tested *in vitro* by mixing WiDr cells and WiDr/CD cells at various ratios and examining both the growth rate (Fig. 4*A*) and the sensitivity to 5FCyt (Fig. 4*B*) in the mixed

Table 1. Incorporation of [6-^{3}H]5FCyt metabolites into RNA and DNA

Type	Tumor	dpm/g of tumor RNA	dpm/g of tumor DNA
WiDr	1	497	114
	2	719	193
WiDr/CD	1	22,594	649
	2	40,653	1160

Tumor-bearing mice were dosed with 500 mg of [6-^{3}H]5FCyt/kg (50 μCi per mouse). Tumors were derived from either WiDr cells or WiDr/CD cells. Twenty-four hours after dosing, tumors were removed and radioactivity was determined in the RNA fraction and DNA fraction.

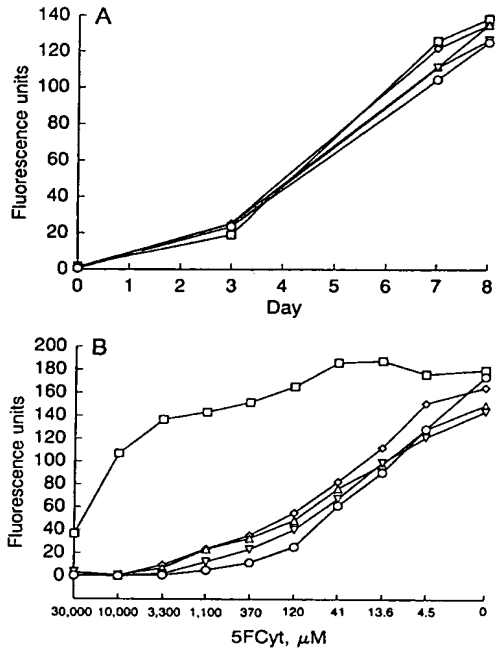

FIG. 4. *In vitro* neighbor cell killing. WiDr and WiDr/CD cells were mixed and plated in 96-well dishes at the following respective ratios: 100:0 (□), 67:33 (◇), 50:50 (△), 33:67 (▽), and 0:100 (○). Each point represents an average of 8 or 16 individual points (SE bars are not included, for clarity). There was no statistical difference in the growth rate of any of the cell mixtures (*A*). Sensitivity to 5FCyt (*B*) in all the cell mixtures was similar except for the cell mixture composed of 100% WiDr cells, which was statistically different from all the other mixtures ($P \leq 0.01$).

cell populations. During this experiment, the absolute cell number and the growth rate of all the untreated mixed cell populations were identical (Fig. 4*A*).

As expected, the cell population of 100% WiDr cells/0% WiDr/CD cells was relatively insensitive to 5FCyt ($IC_{50} \approx$ 22,000 μM) (Fig. 4*B*). However, all the mixed cell populations containing WiDr/CD cells were relatively sensitive to 5FCyt. Mixtures in which 33–100% of the population were WiDr/CD cells exhibited similar sensitivity patterns to 5FCyt ($IC_{50} \approx 25$ μM). These data suggest that one-third of a cell population expressing CD and converting 5FCyt to 5FUra can generate sufficient 5FUra to inhibit the growth of the neighboring two-thirds of the population not expressing CD. The density of the cell population in these experiments indicates that cell–cell contact is not required for this effect. It is therefore concluded that liberated 5FUra is responsible for this neighboring cell killing effect and cell junctions (i.e., gap junctions) are not essential. The significant neighboring cell killing effect generated by the combination of CD expression and 5FCyt treatment observed in these studies was not observed by Mullen *et al.* (11). We have no definitive explanation for this discrepancy.

Neighbor Cell Killing and Antitumor Activity *in Vivo*. Nude mice were implanted s.c. with mixtures of WiDr/CD and WiDr cells at ratios of 100:0, 33:67, 17:83, 8:92, 4:96, 2:98, and 0:100. By day 7, there was 100% tumor yield (i.e., 70 tumors resulting from 70 injections) (Fig. 5). Eight days after implantation, dosing with 500 mg of 5FCyt per kg of body weight began in 5 of the 10 mice in each set. Dosing was approximately once a day until day 22, at which time dosing was ≈3 times a week until day 63. 5FCyt did not produce any overt toxicity in the animals as measured by weight gain compared with control animals. Fig. 5 illustrates the tumor sizes for individual mice at days 7, 27, 48, and 69. Animals were sacrificed when the tumors exceeded 2 g.

In animals receiving no 5FCyt, the average growth rates for all the sets of tumors were statistically identical (Fig. 6*A*). These data are consistent with the *in vitro* growth rate for mixtures of WiDr and WiDr/CD cells (Fig. 4*A*). Due to the excessive size of the tumors in animals not receiving 5FCyt, by day 69, 33 of 35 animals had to be killed (Fig. 5).

In mice receiving 5FCyt, there was no antitumor effect on tumors composed of 0% WiDr/CD cells/100% WiDr cells (Figs. 5 and 6*B*). Very significant tumor regressions were detected in all tumor xenografts containing WiDr/CD cells (Figs. 5 and 6 *C–E*). Tumor cures occurred in all mixtures except 2% WiDr/CD cells/98% WiDr cells. (Cure is defined as no detectable tumor maintained until day 175, at which time the animals were sacrificed. Dosing stopped on day 63.)

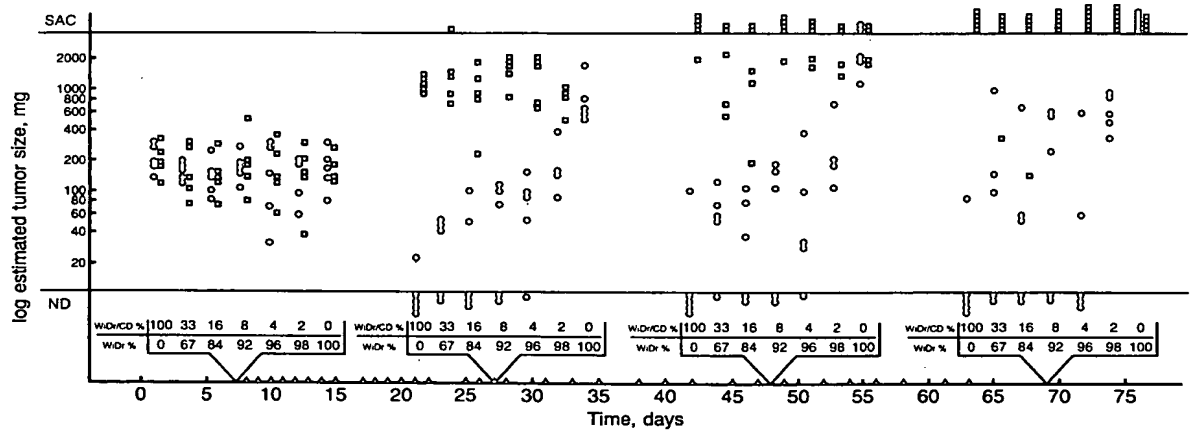

FIG. 5. Tumor weights of mixed-cell tumor xenografts. WiDr and WiDr/CD cells were mixed together at the indicated ratios and injected s.c. into nude mice on day 0. On day 7, tumor weights were estimated and the animals were separated into a nontreatment group (□) and a treatment group (○). On day 7, dosing was initiated with 500 mg of 5FCyt per kg of body weight. Dosing is indicated by the arrowheads on the time axis. Tumor sizes are represented on days 7, 27, 48, and 69. The line marked SAC on the tumor-weight axis indicates that the animal had been sacrificed due to the size of the tumor. The line marked ND (not detected) on the tumor-weight axis indicates that there was no visible tumor.

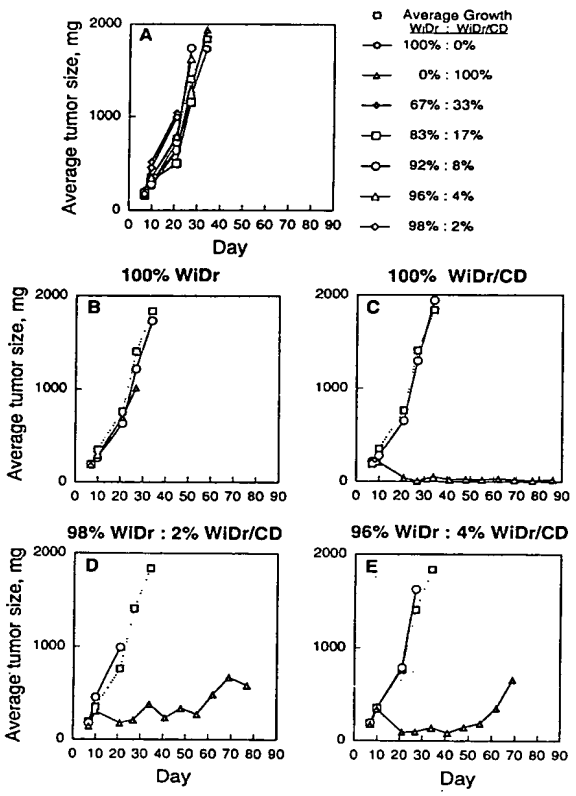

FIG. 6. Average tumor weights of mixed-cell tumor xenografts from Fig. 5. (*A*) Tumor weights in the untreated animals. The growth rates of these tumors were statistically identical ($P \geq 0.344$). The key to *A* is provided in the figure. Since these growth rates were statistically identical, the average growth rate was determined (□). Each group represents a group of five mice and averages were only taken if the group remained at $n = 5$. (*B–E*) Growth rates for tumors composed of 100% WiDr cells/0% WiDr/CD cells (*B*), 0% WiDr cells/100% WiDr/CD cells (*C*), 98% WiDr cells/2% WiDr/CD cells (*D*), and 96% WiDr cells/4% WiDr/CD cells (*E*). In each, ○ and △ represent the tumor growth rates in untreated and 5FCyt-treated animals, respectively; □, average growth rate determined for all the mixtures in the untreated animal group (see *A*). For *B*, average tumor sizes in the treated and untreated groups were statistically identical ($P \geq 0.37$) at any given day. For *C–E*, average tumor sizes in the treated and untreated groups were statistically identical ($P \geq 0.46$) on day 7 (day 7 is start of treatment) but were significantly different from day 21 to the end of the experiment (*C*, $P \leq 0.0023$; *D*, $P \leq 0.087$; *E*, $P \leq 0.087$).

Some tumors began to rebound after day 55 despite the fact that dosing was maintained until day 63 (Figs. 5 and 6). This rebound is presumably due to the elimination of all CD-expressing cells in the tumor at the time of rebound.

Taken collectively, these data indicate that 5FCyt is metabolically converted to 5FUra in cells expressing CD. Subsequent cellular toxicity results from the well-established mechanisms involving TS inhibition and incorporation into RNA by 5FUra anabolites. These mechanistic studies support the use of CD and 5FCyt as part of an enzyme/prodrug combination in gene therapy protocols.

The data demonstrate that only a very small percentage of tumor cells in a solid tumor mass need to express CD to generate a significant antitumor effect. This results from both the significant production of 5FUra in CD-positive tumor cells and the ability of 5FUra to diffuse into neighboring cells, presumably by nonfacilitated diffusion (22). Cell–cell contact is not needed for this effect.

The data suggest that to achieve a significant therapeutic benefit, gene transfer efficiencies of only 1–5% may be needed in virus-directed enzyme/prodrug gene-therapy protocols using the enzyme/prodrug combination of CD and 5FCyt. Required gene transfer efficiencies may actually be less than this if the transduced cell population is permitted to expand before the prodrug is administered. Preliminary data strongly suggest that gene transfer efficiencies at this 1–5% level are achievable with high-titer replication-defective retroviral vectors.

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